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The *Borrelia hermsii* Factor H Binding Protein FhbA Is Not Required for Infectivity in Mice or for Resistance to Human Complement *In Vitro*

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The primary causative agent of tick-borne relapsing fever in North America is *Borrelia hermsii*. It has been hypothesized that *B. hermsii* evades complement-mediated destruction by binding factor H (FH), a host-derived negative regulator of complement. *In vitro*, *B. hermsii* produces a single FH binding protein designated FhbA (FH binding protein A). The properties and ligand binding activity of FhbA suggest that it plays multiple roles in pathogenesis. It binds plasminogen and has been identified as a significant target of a B1b B cell-mediated IgM response in mice. FhbA has also been explored as a potential diagnostic antigen for *B. hermsii* infection in humans. The ability to test the hypothesis that FhbA is a critical virulence factor *in vivo* has been hampered by the lack of well-developed systems for the genetic manipulation of the relapsing fever spirochetes. In this report, we have successfully generated a *B. hermsii fhbA* deletion mutant (the *B. hermsii* YORΔ*fhbA* strain) through allelic exchange mutagenesis. Deletion of *fhbA* abolished FH binding by the YORΔ*fhbA* strain and eliminated cleavage of C3b on the cell surface. However, the YORΔ*fhbA* strain remained infectious in mice and retained resistance to killing *in vitro* by human complement. Collectively, these results indicate that *B. hermsii* employs an FhbA/FH-independent mechanism of complement evasion that allows for resistance to killing by human complement and persistence in mice.

Tick-borne relapsing fever (TBRF) is a significant health concern in regions of the world where it is endemic. It is caused by species in the genus *Borrelia* and is transmitted by *Ornithodoros* ticks (1). TBRF is among the leading causes of febrile illness in certain regions of Africa, exceeding even that of malaria (2, 3, 4, 5). In parts of Tanzania, estimates suggest that 40% of children under the age of 1 will experience at least one bout of relapsing fever (2). In the Americas, TBRF occurs most commonly as outbreaks. Cases in the United States cluster in high-elevation, coniferous forests of the West where the primary etiological agents are *Borrelia hermsii* and *Borrelia turicatae* (6–10). TBRF manifests with recurring episodes of high fever that coincide with the emergence of high-density (10^4 to 10^8 spirochetes ml^{-1} blood), antigenically distinct populations of spirochetes (11). Once infected, the TBRF spirochetes rapidly disseminate to distal tissues, organs, and the central nervous system (12).

The ability of *B. hermsii* to establish infection and persist requires that effective strategies be in place for complement evasion. Several recent reviews have summarized the contribution of factor H (FH) binding in complement evasion by spirochetal pathogens (13, 14). FH is a central negative regulator of the alternative complement pathway (15). It serves as a cofactor for factor I (FI)-mediated cleavage of C3b, inhibits formation of the C3 convertase complex, and accelerates decay of preformed complex (16, 17). Collectively, these processes decrease C3b amplification. *B. hermsii*, the focus of this study, binds FH via its FhbA protein (18). FhbA has also been demonstrated to bind FH-related protein 1 (FHR-1) and plasminogen (19). In contrast to *Borrelia burgdorferi*, a causative agent of Lyme disease, which produces as many as five different FH binding proteins (20–23), FhbA is the sole FH binding protein of *B. hermsii* identified to date (18). FhbA is a 24-kDa, surface-exposed lipoprotein (18, 19) that is encoded by a gene carried on a genetically stable linear plasmid of 200 kb (lp200). With the exception of high-passage-number derivatives of some strains, FhbA is produced by all strains during *in vitro* cultivation. Phylogenetic analyses of FhbA have revealed the existence of two distinct FhbA variants (FhbA1 and FhbA2), both of which bind FH (24, 25). The molecular basis of the FhbA-FH interaction has been investigated using truncation analyses and site-directed mutagenesis (19, 24, 26). In light of the universal distribution of *fhbA* among low-passage-number *B. hermsii* isolates, its expression during infection in humans (23), and its ability to bind serum and complement regulatory proteins, we and others have hypothesized that FhbA is an important virulence factor that plays an essential role in *B. hermsii*'s ability to survive and thrive in blood (18, 19, 25, 27).

While several spirochete species have been shown to bind FH, a clear correlation between FH binding and serum resistance *in vivo* has been demonstrated for only a few species (28–32). Primarily due to the lack of well-developed genetic manipulation systems for the TBRF spirochetes, the potential role that FhbA plays in serum resistance *in vivo* has not been assessed. In this report, a *B. hermsii* YOR *fhbA* deletion mutant was generated and used to test the hypothesis that FhbA is a critical virulence

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factor required for survival in the blood of infected mammals. The data indicate that FhbA and FH binding are not required for complement evasion *in vivo*, suggesting that *B. hermsii* employs yet-to-be-identified FH-independent mechanisms for complement resistance. In addition, this study represents an important technological step forward, as it is the first report to describe the inactivation of a *B. hermsii* virulence factor and subsequent analysis of the resulting mutant in an animal model.

MATERIALS AND METHODS

Bacterial strains and cultivation. *B. hermsii* strain YOR is a well-characterized genomic group II isolate (9, 18, 26). *B. hermsii* YOR and all derivative strains were cultivated in Barbour-Stoenner-Kelly (BSK)-H complete medium supplemented with 12% rabbit serum (37°C; 5% CO_2) in sealed bottles. Where appropriate, kanamycin (200 μg ml^{-1}) was included in the medium. The *B. hermsii* YOR::*kan gfp* strain (33), which was generated in an earlier study and served as a control, carries a selectable marker on lp200 identical to that of the YORΔ*fhbA* strain but retains *fhbA* (33). *Borrelia andersonii* MOD-1 (34, 35), a species in the *B. burgdorferi sensu lato* complex, served as the control for the C9 deposition assays and was cultivated in BSK-H medium with 6% rabbit serum. *Escherichia coli* strains employed for cloning purposes are described in detail below as appropriate.

Vector construction and electroporation. Generation of a *B. hermsii* YOR *fhbA* deletion mutant was achieved through allelic exchange mutagenesis using a suicide vector. This vector was derived from the pFAEV3 vector which was originally generated to integrate a *kan-gfp* cassette into a noncoding region of the 200-kb linear plasmid (lp200) of *B. hermsii* immediately downstream of *fhbA* (33). To construct the pFAEV3Δ*fhbA* plasmid, *fhbA* was excised from pFAEV3 using NheI. The construct was then religated to yield the pFAEV3Δ*fhbA* plasmid. The plasmid was propagated in *E. coli* NovaBlue cells (Novagen) and purified with a HiSpeed plasmid midi kit according to the manufacturer's protocol (Qiagen). Prior to transformation, the pFAEV3Δ*fhbA* plasmid was linearized with AhdI and PflFI restriction enzymes. Electroporation conditions and limiting dilution techniques to obtain clonal populations were as previously described (33). Growth in BSK with kanamycin selected for transformants. Proper integration into lp200 was confirmed by PCR screening. Green fluorescent protein (GFP) production was confirmed by fluorescence microscopy.

Assessment of *in vitro* growth and plasmid composition. *B. hermsii* growth curves were determined as previously described (33). In brief, equal numbers of actively growing cells were inoculated into fresh medium (in triplicate; three independent biological replicates) and maintained at 37°C under 5% CO_2, and cell counts were conducted daily (7 days) using dark-field microscopy. The average number of cells in 10 fields of view (×400 magnification) were determined at each time point and averaged. The plasmid content of each strain was assessed using pulsed-field gel electrophoresis (PFGE) as previously described (36) using conditions optimized for separation of linear DNA molecules between 5 and 200 kb in size. DNA was visualized by ethidium bromide staining.

Immunoblotting and ALBI assays. *B. hermsii* cell lysates were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions in 15% precast Criterion gels (Bio-Rad). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes by electroblotting using standard methods. Immunoblotting and FH affinity ligand binding immunoblotting (ALBI) assays were performed as previously described (29). In brief, for immunoblotting analyses, blots were screened with previously generated mouse anti-FhbA or mouse anti-FlaB antisera (18) at dilutions of 1:1,000 and 1:400,000, respectively. Peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (1:40,000; Pierce) served as the secondary antibody. For ALBI analyses, blots were incubated with purified human FH (5 ng μl^{-1}), and bound FH was detected with goat anti-human FH antiserum (1:1,000; Calbiochem) and peroxidase-conjugated rabbit anti-goat IgG (Pierce; 1:40,000). Detection of secondary antibody was achieved using the SuperSignal West chemiluminescent substrate (Pierce). Throughout this study, all assays involving FH were conducted using purified human FH (Complement Tech). Since the FH is purified directly from human serum, it possesses all-natural posttranslational modifications. At no point was recombinant FH used in this study. Henceforth, purified human FH is referred to simply as FH. Note that all images of immunoblots and FH ALBI assays were cropped to eliminate blank space from the figures.

Surface presentation of FhbA. To determine if FhbA is produced and properly presented on the bacterial surface, proteinase K digestion analyses were performed. Cells (3.5×10^8) from log-phase cultures were collected by centrifugation (5,000 × *g*, 20 min, 4°C), washed with phosphate-buffered saline (PBS), and resuspended in PBS with or without proteinase K (0.2 mg ml^{-1}). Following incubation for 1 h at room temperature, digestion was inhibited with the addition of phenylmethylsulfonyl fluoride (PMSF) (5 μg ml^{-1}). Cells were collected, and FhbA expression at the cell surface was detected by immunoblotting procedures as described above.

Adsorption of FH to viable *B. hermsii* cells. To determine if FH binds to the spirochetal surface, adsorption assays were performed and FH binding was assessed by immunoblotting and indirect fluorescent-antibody assay (IFA) approaches. Cells (1×10^6) were harvested from mid-log-phase cultures by centrifugation, washed with cold PBS, and suspended in 100 μl PBS (supplemented with 1 mM $MgCl_2$ and 0.15 mM $CaCl_2$). FH (0.5 mg ml^{-1}) was added, and the mix was incubated for 1 h at room temperature. Following three washes with PBS, cells were recovered. A portion of the cells were solubilized, fractionated by SDS-PAGE, transferred to membranes, and screened with goat anti-human FH antiserum as described above. To perform IFA to detect FH on the *B. hermsii* cell surface, aliquots of the cells were adjusted to a density of 7.0×10^6 spirochetes ml^{-1}, spotted onto slides (10 μl, Superfrost Plus; Fisher), and air dried. Nonspecific antibody binding was prevented by blocking with 3% bovine serum albumin (BSA) in PBS-0.05% Tween (PBS-T), and then the slides were screened with goat anti-human FH antiserum at a dilution of 1:5,000. The slides were washed three times with PBS-T and then incubated with Alexa Fluor 568-conjugated rabbit anti-goat IgG (1:200; Invitrogen). After a final wash, coverslips were mounted using ProLong Gold antifade reagent (Invitrogen), and the slides were assessed using fluorescence microscopy. Ten fields of view at ×400 magnification were assessed for each strain.

C3b cleavage assay. C3b cleavage assays were performed as previously described (29). Briefly, cells (1×10^6) were harvested from mid-log-phase cultures by centrifugation, washed with PBS, suspended in PBS (with 10 mM $MgCl_2$; 50 μl), and incubated with or without FH (2 μg ml^{-1}; 37°C; 1 h). After three washes, the cells were suspended in PBS containing 10 mM $MgCl_2$, human FI (150 ng; Calbiochem), and human C3b (250 ng; Complement Tech) and incubated for 2 h at 37°C. Cells were pelleted by centrifugation and the supernatants mixed with SDS-PAGE sample buffer, fractionated by SDS-PAGE, and transferred to PVDF membranes by electroblotting. The blots were screened with goat anti-human C3b antiserum (1:800; Complement Tech). Antibody binding was detected as described above. As a positive control for C3b cleavage, purified factor H, FI, and C3b were incubated together in solution with no cells added.

Plasminogen binding assay. Cells were collected, washed with PBS, and immobilized onto enzyme-linked immunosorbent assay (ELISA) plates in carbonate coating buffer (10^6 cells ml^{-1}; 100 μl $well^{-1}$; 4°C; overnight). The wells were washed with PBS-T, and nonspecific binding was inhibited with blocking buffer (PBS with 5% BSA). Plasminogen (10 μg ml^{-1} in blocking buffer; Sigma) was added, and the plates were incubated for 1 h. Controls included coating one set of wells with *B. hermsii* wild-type YOR (YOR-wt) cells and incubating them with either blocking buffer alone or plasminogen alone. Following three washes, bound plasminogen was detected using goat anti-plasminogen antiserum (1:1,000), peroxidase-conjugated rabbit anti-goat IgG (1:40,000), and the addition of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) sub-

TABLE 1 Oligonucleotides used in this study

Primer	Sequence (5′ to 3′)
1	GTCTACTCTATCTGGGATACTAAAGAGC
2	TGTGATTTATTCAATAAAAACAAAAAATTAGATGC
3	TTAAGTTTTTAAATATTCCATTATAGTTTCAAAAAA ATTGTC
4	GAGGCATAAATTCCGTCAGC
5	GTCCACACAATCTGCCCTTTC
6	GCATTAAGTAAGCGGTTTGTGAGC

strate, followed by measuring the absorbance at 405 nm. All binding assays were done in triplicate with three independent biological replicates.

Serum sensitivity assay. The serum sensitivity of each strain was assessed using cells harvested from log-phase cultures. The cells were incubated in 50% or 100% normal human serum (NHS) (Valley Biomedical) or heat-inactivated (56°C; 30 min) human serum (HIS) at 37°C. After 2, 6, and 24 h, cell viability was assessed using the BacLight LIVE/DEAD kit (Invitrogen) and fluorescence microscopic counts of cells in 10 fields under ×400 magnification. Methods for staining and microscopy were as described in earlier publications (37). Data are presented as percent survival, calculated as follows: (average number of intact cells/number of intact and disrupted cells per 10 ×400-magnification fields of view) × 100. *B. andersonii*, a spirochete belonging to the *Borrelia burgdorferi sensu lato* complex (35) that is serum sensitive, served as a control for complement-mediated killing.

Infectivity analyses. The impact of deleting *fhbA* on the ability of *B. hermsii* to infect mice and survive in blood was assessed using C3h/HeJ mice. Mice ($n = 5$ per group; 3 independent experiments) were needle inoculated with each strain (5×10^4 in BSK-H medium), and blood samples were collected (tail nick) at 0, 3, 4, 7, 10, 14, 18, and 42 days postinfection. To prevent clotting, the blood was diluted 10-fold with 0.15 M trisodium citrate. The presence of spirochetes was assessed by direct examination of blood (diluted 1:20 with PBS) using dark-field microscopy. Data are presented as the average number of spirochetes counted per ×400-magnification field of view (10 fields × 20 [dilution factor]). Infectivity was also assessed by cultivation. Undiluted blood (6 μl) from each collection time point was added to BSK-H medium (supplemented with 12% rabbit serum, 50 μg ml^{-1} rifampin, 20 μg ml^{-1} fosfomycin, and 2.5 ng ml^{-1} amphotericin B) and incubated at 37°C. Growth was monitored by dark-field microscopy and scored as plus or minus. All samples cultivated from the blood of individual mice were analyzed by PCR using *fhbA*-specific primers (Table 1). All animal experiments performed as part of this study were conducted following the *Guide for the Care and Use of Laboratory Animals* (eighth edition) and in accordance with protocols peer reviewed and approved by Virginia Commonwealth University Institutional Animal Care and Use Committees.

Serological analyses. Terminal bleeds were conducted at 6 weeks postinoculation, and serum was harvested using standard methods. The IgG response to infection was quantified using ELISAs. ELISA plates were coated with a suspension of wild-type *B. hermsii* YOR cells and screened with serial dilutions of serum collected from each mouse. All methods were as previously described (37).

To determine if anti-FhbA antibodies developed as a result of infection, immunoblot analyses were performed. Recombinant FhbA was fractionated by SDS-PAGE, transferred to a PVDF membrane, and screened with pooled sera collected 4 weeks postinfection from mice infected with each *B. hermsii* strain or from uninfected mice. Methods for detecting anti-FhbA-specific IgG are described above.

Microscopic analyses. All microscopy was performed with an Olympus BX51 microscope fitted with a DP71 camera (Olympus). Dark-field and fluorescence micrographs were recorded with DP Controller 3.11.267 software (Olympus).

RESULTS

Generation and characterization of a *B. hermsii* YOR *fhbA* deletion mutant. The *fhbA* gene of the *B. hermsii* YOR isolate was deleted by allelic exchange using the pFAEV3Δ*fhbA* suicide vector. The replacement of *fhbA* in the *B. hermsii* lp200 plasmid with the kanamycin resistance (*kan*)-*gfp* gene cassette was verified by PCR (Fig. 1A), and fluorescence microscopy served to confirm expression of the integrated *gfp*-containing cassette in both the *B. hermsii* YORΔ*fhbA* and YOR::*kan gfp* strains (Fig. 1). The YORΔ*fhbA* and YOR::*kan gfp* (control) strains were found to have growth rates similar to that of the wild-type parental strain (data not shown). Vectors were also constructed for the purpose of complementing the *fhbA* deletion; however, efforts to obtain complemented strains proved unsuccessful. As detailed below, the inability to generate a complemented strain proved to be irrelevant for this study, since deletion of *fhbA* had no discernible effect on phenotype.

Plasmid-specific PCR has served as the approach of choice for assessing plasmid content of *B. burgdorferi* after electroporation (38). However, the complete sequence of the plasmid component of *B. hermsii* is not available and thus, as an alternative, plasmid content was assessed by pulsed-field gel electrophoresis (Fig. 1C). While there are inherent limitations in this approach (potential for comigrating plasmids and difficulty resolving circular plasmids), no obvious differences in the plasmid content of several clones of the *B. hermsii* wild-type YOR (YOR-wt), YORΔ*fhbA*, and YOR::*kan gfp* strains were observed. To keep the study size manageable and to minimize animal usage, infectivity analyses were conducted with a single clone of each strain that had plasmid profiles and growth rates consistent with the parental wild-type strain.

FhbA production and presentation on the cell surface. Immunoblotting analyses using anti-FhbA antisera revealed that FhbA was produced by the *B. hermsii* YOR-wt and YOR::*kan gfp* strains but not the YORΔ*fhbA* strain (Fig. 2A). Expression levels were not influenced by temperature, as equivalent amounts of FhbA were produced by cultures maintained at 25 and 37°C (data not shown). We also verified that FhbA produced by the YOR::*kan gfp* strain is presented on the cell surface in a manner similar to wild-type cells using proteinase K digestion assays. Consistent with surface exposure, proteinase K degraded FhbA in the *B. hermsii* YOR-wt and YOR::*kan gfp* strains (Fig. 2A) but not FlaB (negative control), an inner membrane-anchored protein.

FH binding assays. To determine if deletion of *fhbA* eliminates FH binding, cell lysates of each strain were fractionated by SDS-PAGE and tested for FH binding using ALBI assays (18, 27). All strains bound FH except the YORΔ*fhbA* strain (Fig. 2B, bottom). An identical blot was screened with anti-FlaB to verify equivalent loading of the gels (Fig. 2B, top). To determine if FH can interact with the *B. hermsii* cell surface in an FhbA-independent manner, two independent approaches were employed. Pulldown assays were performed by incubating mid-log-phase cultures of each strain with FH. The cells were pelleted and separated by SDS-PAGE, transferred to membranes, and then screened with anti-human FH or anti-FlaB antiserum (pulldown control). FH bound to *B. hermsii* YOR-wt and YOR::*kan gfp* strains but not the YORΔ*fhbA* strain (Fig. 2C). FH binding to the cell surface was also assessed using IFA, as detailed above. FH was readily detected on the surface of the *B. hermsii* YOR-wt and YOR::*kan gfp*r strains but

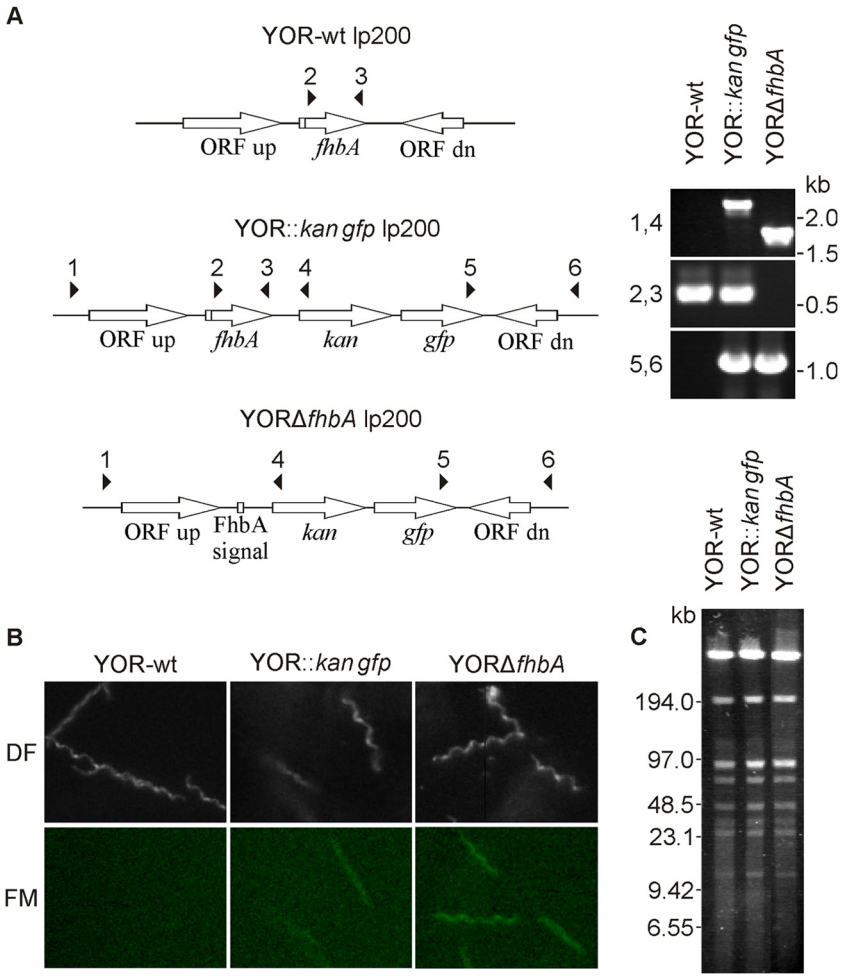

FIG 1 Allelic exchange mutagenesis. The schematics in panel A (left) depict the gene organization near *fhbA* on lp200 before and after allelic exchange mutagenesis. The upstream and downstream open reading frames (ORFs) that are adjacent to *fhbA* on plasmid lp200 are indicated in the figure as "ORF up" and "ORF dn," respectively. Primer target sites (black arrowheads), with primer numbers indicated above each arrowhead, are shown. Primer sequences can be found in Table 1. The primers are numbered in the figure as they are in Table 1. To the right of the schematic, the results of PCR analyses conducted to confirm proper insertion of the mutagenesis cassettes are shown (ethidium bromide-stained gels). While numerous clones were analyzed, the data presented in panels B and C were obtained with the specific clonal populations that were employed in all subsequent experiments. The criteria used for selecting specific clones for all subsequent work are detailed in the text. In panel B, fluorescence microscopy (FM) was conducted to verify production of GFP (which is encoded in the cassette used for mutagenesis). The accompanying dark-field microscopic images served as a control to show that all cells present on the slide produce GFP. In panel C, plasmid content was assessed by pulsed-field gel electrophoresis, followed by staining with ethidium bromide. Molecular mass markers are shown to the left in kb. All methods are detailed in the text.

not on the YORΔ*fhbA* strain (Fig. 2D). It can be concluded that FH binding to *B. hermsii* is strictly dependent on FhbA and that, as suggested in earlier studies (23, 25), FhbA is the sole FH binding protein of *B. hermsii*.

Analysis of C3b inactivation. In previous studies, it was demonstrated that FH bound to the surface of *B. hermsii* YOR efficiently serves as a cofactor for the factor I-mediated cleavage of C3b (24). To determine if deletion of *fhbA* eliminates the ability of *B. hermsii* to facilitate C3b cleavage, each strain was incubated with human C3b and FI, with and without FH. FhbA-expressing strains cleaved C3b, while the *B. hermsii* YORΔ*fhbA* strain did not (Fig. 3). It can be concluded that FhbA and FH binding are required for C3b degradation and *B. hermsii* cannot degrade C3b in an FH/FI-independent manner.

FhbA expression is not required for plasminogen binding. In addition to FH, *B. hermsii* FhbA binds host-derived plasminogen (19). Binding of plasminogen and its conversion to plasmin may play an important role in invasion and dissemination (39, 40). Plasminogen binding was assessed using an ELISA format. Statistically significant differences in plasminogen binding were not detected among strains, indicating that FhbA is not required for plasminogen binding (Fig. 4).

Analysis of serum sensitivity and complement resistance. To assess the contribution of FhbA and FH binding in complement evasion *in vitro*, each strain was incubated with complement-active NHS or HIS (complement inactive) and cell killing was assessed. *B. andersonii* strain MOD-1 served as a positive control for serum-mediated killing. While *B. hermsii* high-passage-number laboratory strains have been shown to be of intermediate serum sensitivity and thus could have potentially served as controls, *B. andersonii* was selected, because this species displays a high-serum-sensitivity phenotype. The *B. hermsii* YOR-wt, YOR::*kan gfp*,

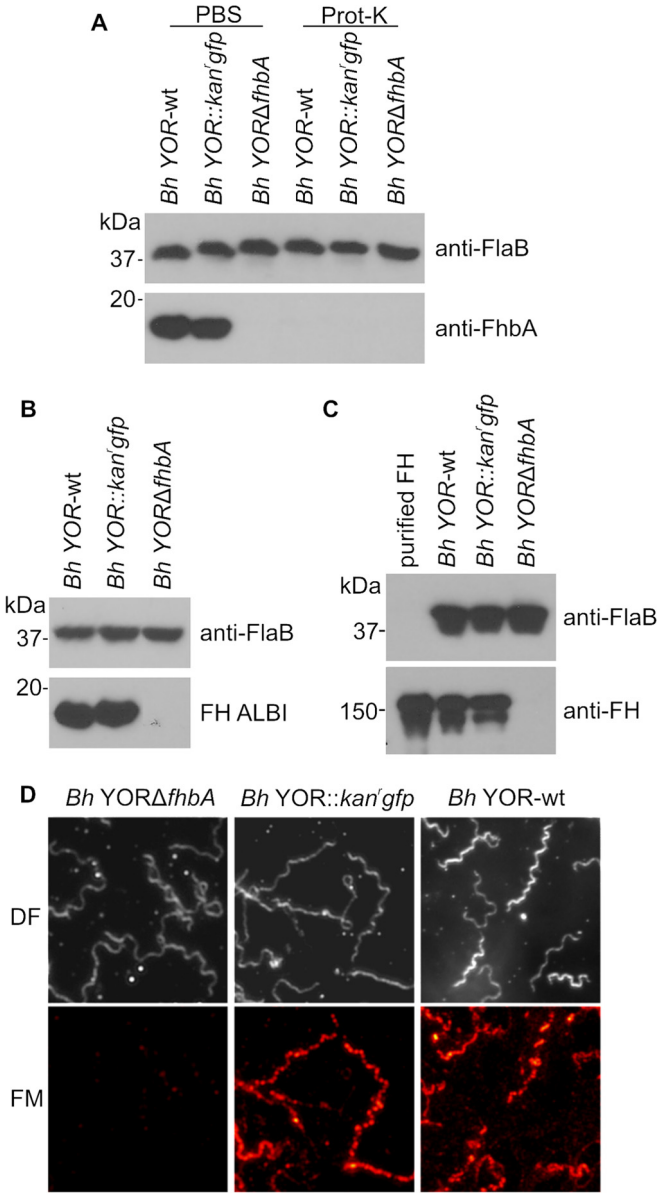

FIG 2 Analysis of FhbA production. (A) To assess production and surface presentation of FhbA by *B. hermsii* wild-type and transformant clonal populations, cells were incubated with or without proteinase K. The cell lysates were fractionated by SDS-PAGE, immunoblotted, and screened with anti-FhbA and anti-FlaB antiserum (to confirm equal loading). (B) FH binding was analyzed using affinity ligand binding immunoblotting (ALBI) assays as detailed in the text. In brief, immunoblots of cell lysates were overlaid with FH, and bound FH was detected using anti-human FH antiserum. A second blot was screened with anti-FlaB antiserum to demonstrate equivalent loading of the cell lysates. (C) The potential requirement for FhbA in FH binding was assessed through whole-cell absorption assays. Cells were harvested by centrifugation, resuspended, and incubated with FH. The cells were recovered, lysed, fractionated by SDS-PAGE, transferred to membranes, and screened with anti-human FH or anti-FlaB antiserum. As described above, anti-FlaB antiserum was used to demonstrate equal loading of protein, and purified FH was loaded in one lane as a control. (D) To determine if FH binds to the surface of each strain, FH was incubated with actively growing cells and binding was detected using goat anti-human FH antibody with Alexa Fluor 568-conjugated anti-goat IgG as the secondary antibody (as detailed in the text). Ten ×400-magnification fields of view were assessed, and no less than three biological replicates were performed for each experiment.

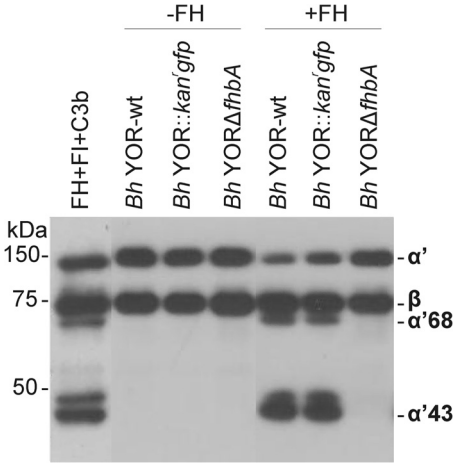

FIG 3 Analysis of C3b cleavage. Cells were incubated with or without FH (as indicated above the lanes), and then purified human FI and C3b were added. As a control for C3b cleavage, FH, FI, and C3b were coincubated in solution in the absence of cells. The samples were solubilized, subjected to SDS-PAGE, transferred to membranes, and screened with anti-C3 antiserum. Expected sizes of the C3b α and β chains, as well as the factor I-mediated C3b cleavage products α′43 and α′68, are indicated to the right of the panel, with molecular mass standards indicated on the left.

and YORΔ*fhbA* strains were serum resistant, even after exposure to 50% human serum for 24 h (Fig. 5). In contrast, *B. andersonii* was killed within 2 h. The *B. hermsii* YOR-wt, YOR::*kan gfp*, and YORΔ*fhbA* strains also proved to be serum resistant when incubated in 100% human serum for 24 h (data not shown). Hence, the data presented here do not support the hypothesis previously put forth by our laboratory that the FhbA-FH interaction is required for serum resistance and infectivity (18, 25) and suggest that yet-to-be-identified FH-independent mechanisms are involved in complement evasion.

FhbA and FH binding are not required for *B. hermsii* to infect mice. To determine if FhbA is required for infectivity and survival in blood, mice were needle inoculated, and relative spirochete numbers in blood were assessed over time. This was accom-

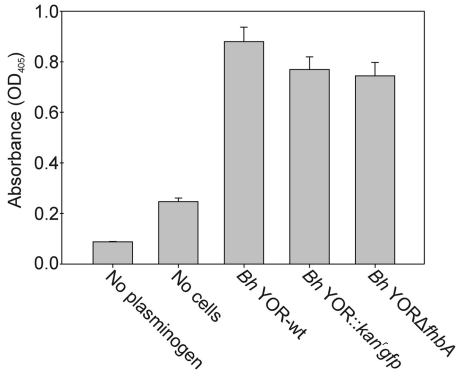

FIG 4 Plasminogen binding assays. An ELISA format was used to measure plasminogen binding. Cells were immobilized into 96-well plates, and plasminogen binding was measured as detailed in the text. Negative controls consisted of wild-type *B. hermsii* with no plasminogen added and BSA-coated wells with plasminogen added. All assays were performed in triplicate, with the standard deviations indicated by error bars. The data shown are representative of 3 independent experiments.

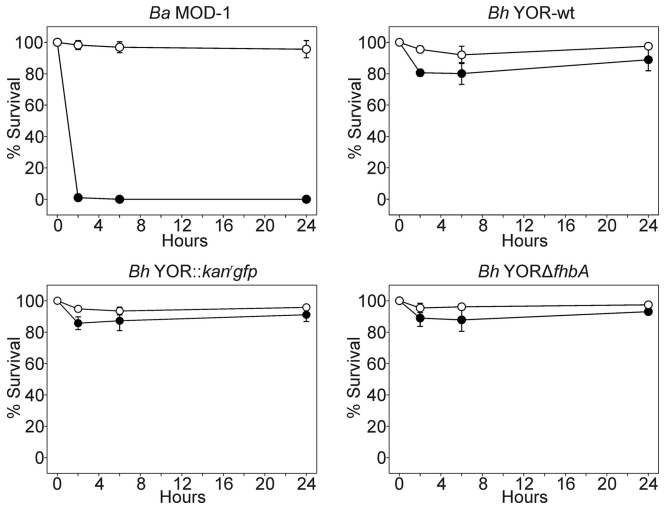

FIG 5 Serum sensitivity analyses. *B. andersonii* MOD-1 (serum-sensitive control) and each *B. hermsii* strain were incubated with 50% normal human serum (NHS; closed circles) or 50% heat-inactivated human serum (HIS; open circles). Percent survival was assessed after 2, 6, and 24 h using BacLight LIVE/DEAD staining and fluorescence microscopy. Mean percent survival (number of intact cells divided by the total number of intact and morphologically disrupted cells × 100) was calculated from analysis of 10 ×400-magnification fields per strain per time point. The data shown are the average counts obtained from 10 fields of view. Error bars indicate the standard deviations. The data presented are representative of 3 independent experiments.

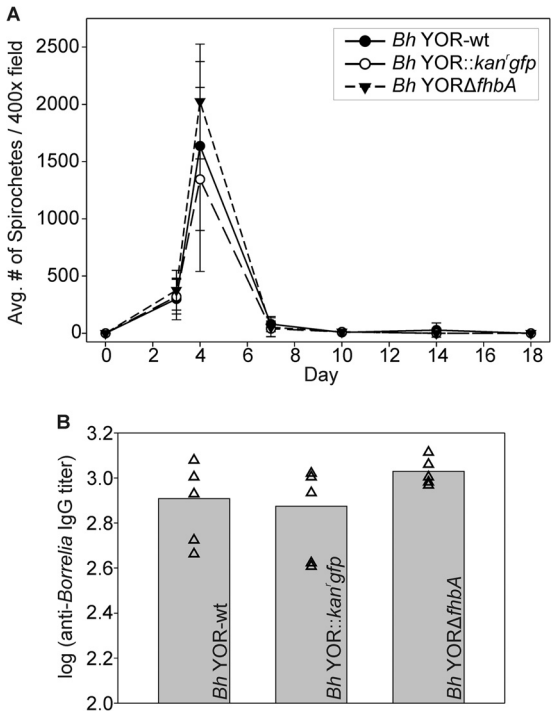

FIG 6 Analysis of murine infectivity and seroconversion. Mice ($n = 5$ per group) were infected with each strain by needle inoculation. At days 3, 4, 7, 10, 14, and 18 postinoculation, peripheral blood samples were obtained and assessed for the presence of spirochetes by dark-field microscopy (A). Data are presented as the average number of spirochetes counted in 10 ×400-magnification fields of view × the dilution factor of 20. Error bars indicate the standard deviation. The infectivity studies were conducted a total of three times by two different members of our laboratory using different freeze back stocks to generate the cultures. For panel B, ELISAs were performed using serum harvested from mice 4 weeks postinoculation to determine the anti-*B. hermsii* YOR titers elicited by each infecting strain (as indicated). Geometric means (gray bars) were determined from titers calculated for each mouse (triangles) at one-third of the max optical density. All ELISAs were conducted in triplicate. The bar graphs represent the averages from the titers, determined for each group of 5 mice. Individual data points for each mouse are shown.

plished by visual counting of spirochetes in blood samples using dark-field microscopy. All strains were infectious and reached comparable densities in the blood (Fig. 6A). A single spirochetemic peak was observed at day 4. Although additional spirochetemic peaks were not observed, cultivation of blood samples revealed that the *B. hermsii* YOR-wt, YOR::*kan gfp*, and YOR*ΔfhbA* strains persisted in blood up to 14 and 18 days in individual animals (Table 2). Consistent with this, serological analyses revealed that all mice developed anti-*B. hermsii* IgG titers (Fig. 6B). To verify the *fhbA* genotype of the spirochetes reisolated from blood, PCR analyses were performed using an *fhbA* primer set. Amplicons were obtained from reisolated *B. hermsii* YOR-wt and YOR::*kan gfp* isolates but not from YOR*ΔfhbA* isolates (Fig. 7A). To determine if FhbA was produced by each strain during infection, antiserum from each mouse was used to screen immunoblots of r-FhbA. Anti-FhbA antibody was detected in sera collected 4 weeks postinfection from *B. hermsii* YOR-wt- and YOR::*kan gfp*-infected mice but not from mice infected with the YOR*ΔfhbA* strain (Fig. 7B).

DISCUSSION

The FhbA protein of *B. hermsii* binds host-derived proteins that are important in complement regulation, including FH, FHR-1, and plasminogen (18, 19). Based on this and its universal distribution among low-passage-number isolates, it has been postulated that FhbA is a virulence factor required for complement evasion (19, 25). An earlier study revealed that the *B. hermsii* REN strain (which lacks *fhbA*) displayed greater serum sensitivity than other isolates that produce FhbA (25). However, because the REN used in the cited study had been passaged *in vitro* >100 times, its increased sensitivity to serum could have resulted from other genotypic or phenotypic changes that can occur during extended cultivation. The ability to directly assess FhbA's role in complement evasion has been hampered by inherent difficulties of genetically manipulating the TBRF spirochetes. The goal of this study was to determine if FhbA and hence FH binding is required for *B. hermsii* to evade complement-mediated killing *in vitro* and to infect mice and persist.

To address these questions, *fhbA* was deleted using allelic exchange mutagenesis to yield the YOR*ΔfhbA* strain. This deletion mutant displayed growth kinetics similar to those of the parental wild-type strain, and PFGE did not reveal the loss of plasmids or genomic rearrangements. Using several different approaches to assess FH binding, it was demonstrated that deletion of *fhbA* results in the complete loss of FH binding by the *B. hermsii* YOR*ΔfhbA* strain, consistent with FhbA being the sole FH binding protein of *B. hermsii* (18). Surprisingly, deletion of *fhbA* had no impact on the ability of the YOR*ΔfhbA* strain to survive in human serum or to establish infection and persist in mice. All mice developed a single spirochetemic peak during days 3 and 4 postinoculation. While relapses were not evident, spirochetes were readily cultivated from blood collected up to 2 weeks postinoculation. Hence, the *fhbA* mutant was able to survive in blood, albeit at low

TABLE 2 Summary of blood culture results from mice infected with wild-type or transformant *B. hermsii* YOR strains

B. hermsii strain	Blood culture result[a] on day:							
	0	3	4	7	10	14	18	42
YOR-wt strain	0/5	5/5	5/5	5/5	5/5	1/5	0/5	0/5
YOR::*kan gfp* strain	0/5	5/5	5/5	5/5	4/5	2/5	0/5	0/5
YORΔ*fhbA* strain	0/5	5/5	5/5	5/5	5/5	3/5	2/5	0/5

[a] Data are shown as number of samples yielding positive culture/total number of samples.

levels, as long as the wild-type strain. It can be concluded that *fhbA* and FH binding are not required for evasion of complement in human serum and in mice.

The properties of the *fhbA* deletion mutant suggest that *B. hermsii* employs FH-independent mechanisms for complement evasion. *B. hermsii* produces a serine protease (BhpA) that is abundantly expressed *in vitro* and during infection in mice (41) that could conceivably cleave complement proteins and play a compensatory role in complement evasion in the FhbA deletion mutant. However, in this study, cleavage of C3b by the *fhbA* deletion mutant was not observed, suggesting that BhpA is not directly involved in cleaving this important opsonin. The potential for BhpA to facilitate complement evasion by potentially cleaving other complement regulators has not yet been directly assessed. Proteolytic inactivation of complement would be a novel immune evasion mechanism for spirochetes and should be considered in future studies.

Other TBRF spirochetes have been shown to utilize multiple complement evasion mechanisms. In addition to FH, *Borrelia recurrentis* and *Borrelia duttonii* also bind C4BP, a central regulator of the antibody-mediated classical complement pathway (42, 43). C4BP serves as a cofactor in the FI-mediated cleavage of C4b. However, binding analyses suggest that C4BP does not bind to *B. hermsii* (43) (R. T. Marconi, unpublished data), and hence C4BP binding does not appear to be involved in *B. hermsii* serum resistance. *B. recurrentis*, a louse-borne relapsing fever spirochete, binds C1 esterase inhibitor (C1-INH) via the CihC protein (43).

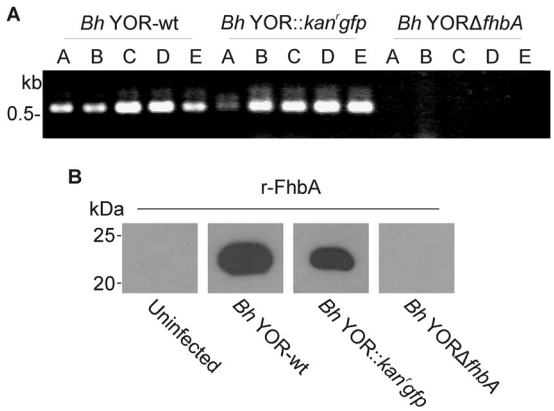

FIG 7 Analysis of the *fhbA* genotype in recovered isolates and anti-FhbA antibody responses in infected mice. Isolates that were cultivated from mice infected with each strain (indicated in the figure) served as the template for PCR amplification of *fhbA*. Panel A depicts the PCR results. The individual mice within each group were designated A through E. In panel B, pooled serum harvested from each infected mouse group was screened for the presence of anti-FhbA IgG using immunoblotting approaches, with recombinant FhbA serving as the immobilized antigen. Serum for an uninfected mouse served as a negative control.

C1-INH is a serine protease inhibitor that contributes to the regulation of both the classical and lectin pathways of complement (44). While the binding of C1-INH cannot be excluded for *B. hermsii*, it would not explain the *in vitro* resistance to the alternative pathway complement-mediated killing displayed by the FhbA deletion mutant.

B. hermsii has been demonstrated to recruit and activate plasminogen on its surface (19, 45), a process that has been demonstrated to facilitate penetration of endothelial cell monolayers (45). A recent study suggests that plasminogen also has a complement regulatory role (inhibitory) (46). Plasminogen binding analyses revealed that the *B. hermsii* YORΔ*fhbA* strain binds plasminogen at levels similar to those of the YOR-wt strain. It can be concluded that FhbA is not required for potentially important functions that are mediated by plasminogen binding.

The demonstration that *B. hermsii* does not require FhbA raises the question as to why FhbA is universal, conserved, and constitutively produced. It is possible that FhbA could have functional roles that were not directly tested here, such as facilitating penetration of endothelial cells and crossing of the blood-brain barrier. Alternatively, FhbA may play an important functional role in the tick. One possibility is that FhbA facilitates complement evasion specifically in ticks during the blood meal. This remains to be tested. A similar hypothesis has been suggested for the FH binding-CspA protein of *B. burgdorferi* (47). Unfortunately, in contrast to *Ixodes* ticks, *Ornithodoros* ticks are not currently commercially available, and hence it was not possible in this study to investigate the potential biological role of FhbA in the tick environment.

In summary, this study is among the first to successfully genetically manipulate a potential virulence factor of a TBRF spirochete and study the mutant strain *in vivo*. FhbA is known to bind to three human-derived ligands: FH, FHR-1, and plasminogen (18, 19). However, deletion of *fhbA* revealed that it is not required for evasion of killing mediated by the alternative complement pathway *in vitro* or for survival in mice when delivered by needle inoculation. It can also be concluded that its function in plasminogen binding is not essential for infectivity. This study suggests that FH/FHR-1-independent mechanisms contribute to *B. hermsii's* ability to evade killing by the alternate complement pathway. Future studies utilizing the *fhbA* deletion mutant will seek to determine if FhbA contributes to other roles during infection and transmission, such as invasiveness, adherence, or survival in ticks. While the functional role of FhbA remains a question, this study represents an important step forward in the development of tools that will facilitate the study of other *B. hermsii* virulence factors *in vivo*.

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